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THE VOGES-PROSKAUER AND CORRELATED REACTIONS OF COLI-LIKE BACTERIA *

MAX LEVINE, J. C. WELDIN, AND BERYL R. JOHNSON

From the Laboratories of the Engineering Experiment Station and the Department of Bacteriology and Hygiene of the Iowa State College, Ames.

It is becoming increasingly apparent that all nonsporing, lactose-fermenting bacteria are not of equal sanitary significance. There is no doubt that a water containing the relatively nonresistant B. coli communis and B. communior, or their very close allies is more objectionable and dangerous than one containing only the more ubiquitous and quite resistant B. aerogenes or B. cloacae. These 2 groups of lactose-fermenting organisms should be differentiated, and studies on rapid methods for their isolation, separation, and identification are sorely needed. It is the primary purpose of this paper to record the results of attempts to hasten the Voges-Proskauer reaction and to point out its apparent correlation with the fuchsin-aldehyd test.

THE FUCHSIN-ALDEHYD REACTION

If an aldehyd is added to a solution of basic fuchsin, decolorized with sulphurous acid, a distinct red to violet color appears usually in less than 1 or 2 minutes. This reaction is accepted by chemists as specific for aldehyds and is universally employed as a group test. In all probability, the red coloration on Endo agar, so characteristic of B. coli, is merely a localized fuchsin-aldehyd test. It should be noted, however, that in the Endo medium, the fuchsin is decolorized with Na_2SO_3 and not with H_2SO_3. At the suggestion of Dr. R. E. Buchanan, this reaction was tried with a number of coli-like organisms to determine if it is of any differential value. All of the organisms studied were isolated from soil. The test was made as follows: To 2 c.c. of a 72-hour culture of the organism in 0.5% carbohydrate-peptone-dipotassium-phosphate solution, were added 2 or 3 drops of basic fuchsin decolorized with sodium sulphite. A slight pink coloration was recorded as negative while a distinct red or cherry color was recorded positive. The results are shown in Table 1.

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TABLE 1

COMPARISON OF METHYL-RED AND VOGES-PROSKAUER REACTIONS FROM GLUCOSE WITH THE FUCHSIN-ALDEHYD TEST

	Fuchsin-Aldehyd Test			
	Glucose		Lactose	
	+	−	+	−
Methyl-Red +	47	0	32	0
Methyl-Red −	7	115	30	25
Voges-Proskauer +	10	114	32	25
Voges-Proskauer −	44	1	30	0

* Basic fuchsin decolorized with sodium sulphite. Figures indicate number of strains.

Of 122 organisms which were alkaline to methyl red in 0.5% glucose, only 7 (5.7%) gave a positive fuchsin-aldehyd test while all (100%) of 47 strains, which were acid to methyl red, were positive for this aldehyd test. Again, among 124 cultures which gave the Voges-Proskauer reaction, only 10 (8.0%) were positive with the fuchsin test as compared with 44 (98.0%) of 45 Voges-Proskauer negative strains.

The correlation between the methyl-red or Voges-Proskauer reaction with the modified fuchsin-aldehyd test is evident. It must not be concluded, however, that the B. aerogenes and B. cloacae groups do not form an aldehyd. In an unpublished study by George G. DeBord of this laboratory, it was observed that the fuchsin-aldehyd test is obtained only in an acid medium. He found that when basic fuchsin is decolorized with sodium sulphite and then acidified, only a faint coloration, if any, is observed. Similarly, the addition of formaldehyd to the reduced fuchsin also failed to produce any definite reaction. If, however, both an acid and formaldehyd were added, the characteristic aldehyd test was obtained.

The failure of the B. aerogenes-cloacae group to give a characteristic aldehyd reaction when so tested may therefore be merely another method of indicating a difference in acidity between the aerogenes-cloacae and the colon group. The difference observed may also throw some light on the significance of the various types of colored colonies occurring on Endo agar. All of 32 true B.-coli strains studied gave a distinct aldehyd test from lactose, while only 30 (54.6%) of 55 B. aerogenes and B. cloacae strains were positive. The aldehyd test from glucose after 3 days' incubation at 37 C. is, therefore, better correlated with the Voges-Proskauer and methyl-red reactions than is the aldehyd test from lactose.

EFFECT OF CONCENTRATION OF CARBOHYDRATE ON VOGES-PROSKAUER AND METHYL-RED REACTIONS

A few simple experiments were made to determine the effect of concentration of sugar and period of incubation on the qualitative methyl red and acetyl-methyl-carbinol reactions; the rapidity with which the test for acetyl-methyl-carbinol may be recognized and the permanancy of the coloration.

Cultures of B. cloacae and B. aerogenes, known to give the Voges-Proskauer reaction, were studied. The organisms were inoculated from 24-hour agar slants into 100 c.c. of 0.5% peptone-dipotassium-phosphate water containing various concentrations of glucose or sucrose (0.5, 1, 2, and 5%), and incubated at 37 C. At intervals of 1, 2, 3, and 5 days, portions were removed and tested for acetyl-methyl-carbinol, and also qualitatively with methyl red.

The methyl-red test was made by adding 0.2 c.c. of the indicator to 5 c.c. of the culture. The reaction could, of course, be recognized immediately. To test for acetyl-methyl-carbinol, 2 or 3 c.c. of the culture were added to an equal volume of 10% KOH, and the mixture was allowed to stand exposed to the air. The presence of the characteristic eosin-like coloration was observed and recorded hourly for 5 hours, and again after 24, 48, and 72 hours. The results are summarized in Table 2.

Methyl-Red Reaction.—It is seen from Table 2 that B. cloacae and B. aerogenes were alkaline to methyl red after 24 hours' incubation, when 0.5% of the carbohydrates were employed, but were acid or in neutral tints with the higher concentrations of sugar. With 1.0% glucose, B. cloacae was alkaline on the 2nd day, while B. aerogenes was acid, but became alkaline on the 3rd day. In a general way, the time required for reversion from an acid to an alkaline reaction increases with the concentration of carbohydrates. With 5% glucose or sucrose, the medium was still acid after 1 month's incubation. The reversion with B. cloacae is more rapid than with B. aerogenes.

The Acetyl-Methyl-Carbinol Test.—It is customary to allow the KOH-culture mixture to stand for 24 hours before recording for the carbinol test, and some investigators do not record until after 48 hours.

This is extremely unfortunate as it results in an unnecessary loss of time. With the 2 organisms mentioned, a faint reaction could be recognized 1 hour after addition of the KOH, and after 5 hours, the coloration was usually as marked and distinct as at the expiration of 24 hours. The color was still recognizable after 2 days, but quite indistinct on the 3rd day. That the reaction may be seen in about 1 hour has been previously observed by Durham.[1] With concentrations of glucose above 1%, the medium is so highly colored that the pink or eosin coloration of the Voges-Proskauer reaction is obscured. As a positive test was obtained after 1 day's incubation at 37 C., the records of the Voges-Proskauer reaction are not included, for brevity and clearness, in Table 2.

TABLE 2

EFFECT OF CONCENTRATION AND PERIOD OF INCUBATION ON THE REACTION WITH METHYL RED

Concentration	B. cloacea and Glucose				B. cloacea and Sucrose				B. aerogenes and Glucose				B. aerogenes and Sucrose			
	Incubation Days				Incubation Days				Incubation Days				Incubation Days			
	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5
0.5%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1.0%	+*	–	–	–	+	–	–	–	+	+	–	–	+	+	+	–
2.0%	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+
5.0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Reactions in the neutral tints included as positive.

It appeared from the preliminary experiment that, if necessary, about 5 hours after the addition of KOH would be a safe time to record for the carbinol reaction as a presumptive test. This was verified by a confirmatory study of 152 strains employing 10 test substances. The results after 5 and 24 hours are indicated in Table 3.

Of 140 strains which gave the Voges-Proskauer reaction from glucose, 130 (92.9%) were positive after 5 hours. A similar result was observed with sucrose, where of 134 positive carbinol tests, 127 (94.8%) were obtained in 5 hours. With other test substances, the number and percentage of positive tests, after 5 hours, were as follows: raffinose, 114 (100%); mannitol, 116 (88.5%); salicin, 86 (82.7%); dulcitol, 11 (78.6%). With galactose and lactose, the 5-hour period was not reliable. These media became very dark on the addition of a strong alkali, thus obscuring the reaction. It is concluded from these

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[1] Jour. Exper. Med., 1901, 5, p. 353.

TABLE 3

COMPARISON OF FIVE HOUR AND TWENTY-FOUR HOUR RECORDS OF TESTS FOR ACETYL-METHYL-CARBINOL

Test Substances	Total Positive Reactions	Positive in 5 Hr.		Positive in 24 Hr. Negative in 5 Hr.	
		No.	%	No.	%
Glucose	140	130	92.9	10	7.1
Galactose	96	53	55.2	43	44.8
Lactose	90	0	0.0	90	100.0
Sucrose	134	127	94.8	7	5.2
Raffinose	114	114	100.0	0	0.0
Mannitol	131	116	88.5	15	11.5
Salicin	104	86	82.7	18	12.3
Dulcitol	14	11	78.6	3	21.4
Dextrin	12	12	100.0	0	0.0
Starch	18	17	94.0	1	0.0

results that the 5-hour period may be employed as a presumptive test for acetyl-methyl-carbinol.

On Accelerating the Carbinol Test.—Hardin ascribes the Voges-Proskauer reaction to the production of acetyl-methyl-carbinol, CH_3 $CHOH.CO.CH_3$, which is an oxidation product of 2: 3 butyleneglycol ($CH_3.CHOH.CHOH.CH_3$). The carbinol becomes further oxidized to diacetyl $(CH_3CO)_2$ on exposure to air, which in the presence of strong KOH reacts with peptone to form an eosin-like compound. The problem of hastening this reaction resolves itself, therefore, into one of finding an oxidizing agent capable of oxidizing acetyl-methyl-carbinol to diacetyl, but which will not form the carbinol from butyleneglycol.

A number of experiments were tried with a culture of B. aerogenes and B. cloacae, organisms known to form acetyl-methyl-carbinol, and B. communior, which does not give the carbinol test as a check. The oxidizers employed were aerated potassium hydroxid, potassium dichromate, potassium chlorate, potassium perchlorate, barium peroxid, calcium hypochlorite, and hydrogen peroxid. As the reaction is more intense and would be more easily observed in sucrose media, which is colorless, this medium was employed in place of glucose.

The 1st test was an attempt to hasten the reaction by using a 10% KOH solution, through which air had been drawn over night. The results with this aerated KOH solution were somewhat better than when plain KOH was used, but not markedly so. We then tried mixing the KOH and culture media and heating for 1 or 2 minutes, but apparently with but slight increase in speed of reaction.

The addition of an oxidizing agent was next tried. Varying dilutions of $K_2Cr_2O_7$ (0.005 = 5.0%) were made in 10% KOH solution. Each concentration of dichromate was tested as follows: Five c.c. were placed in each of 3 test tubes; to 1 was added an equal volume of the culture under examination and the mixture was allowed to stand at room temperature; to the 2nd, an equal volume of the culture was added and the mixture heated for 2 minutes in a boiling water bath, after which it was placed by the side of the 1st tube. To the 3rd tube the culture was added in such a way as to form a layer over the dichromate KOH mixture, thus permitting the formation of a ring test. The tubes were examined at the end of 15 minutes and again after 3 hours. The results are shown in Table 4. A distinct reddening, as compared with a check tube, was recorded as positive.

TABLE 4

EFFECT OF POTASSIUM DICHROMATE ON TEST FOR ACETYL-METHYL-CARBINOL

Concentration $K_2Cr_2O_7$ in %	B. communior			B. cloacae					
	3 Hours			15 Minutes			3 Hours		
	Ordinary Test	Heated	Ring Test	Ordinary Test	Heated	Ring Test	Ordinary Test	Heated	Ring Test
0.005	—	—	—	f	m	—	s	s	—
0.01	—	—	—	f	m	—	s	s	f
0.05	—	—	—	f	m	—	s	s	m
0.1	—	—	—	f	m	—	s	s	m
0.2	—	—	—	f	s	—	s	s	m
0.5	—	—	—	f	s	—	s	s	s
1.0	—	—	—	f	s	f	s	s	s
5.0	—	—	—	f	s	f	v s	v s	v s

f = faint; m = moderate; s = strong; v s = very strong.

As $K_2Cr_2O_7$ imparts a yellow or orange color to the solution, particularly in its higher concentrations, a weak reaction might be masked; consequently, it is not the ideal oxidizing agent for our purpose. Nevertheless, if checks are run for comparison, this reagent may be safely employed after a little practice.

It is seen from Table 4 that a concentration 0.2% or more of potassium dichromate, in 10% KOH solution, when added to a culture and the mixture heated, will accelerate the carbinol test so that a strong reaction may be observed in 15 minutes. The ring test was not found suitable with this reagent.

Among other substances tested were bleaching powder, potassium chlorate, potassium perchlorate, barium peroxid, and hydrogen peroxid. All were capable of accelerating the reaction, but the most consistent and the best results were obtained with hydrogen peroxid.

The first attempts with hydrogen peroxid were very disappointing. When employed in a manner analogous to that in which the potassium bichromate was used, no test could be obtained at all, because of the bleaching action of peroxid. After many futile attempts it was suggested that the hydrogen peroxid be added after the KOH-culture mixture was heated. This procedure was very successful. If 3 c.c. of a 48-hour sucrose-peptone culture is added to an equal volume of 10% KOH, the mixture heated in a boiling water bath for 2 minutes, then 2 or 3 drops of hydrogen peroxid added, the pink coloration of the carbinol test appears in 1 or 2 minutes and will persist for several hours. If an excess of hydrogen peroxid is used, the color will disappear in a very few minutes, or even instantly, if the excess is very great.

This rapid method of testing for acetyl-methyl-carbinol was then tried with more than 100 strains, including B. aerogenes, B. cloacae, B. coli communis, B. communior, etc., and also employing different carbohydrates. With sucrose, mannitol, raffinose, and salicin, substances giving colorless media, the hydrogen-peroxid test as applied above gave as good results as were obtained by allowing the KOH-culture mixture to stand at room temperature for 24 hours. With glucose, however, the use of hydrogen peroxid did not give as good results. The difficulty is probably due to the coloration which develops when the glucose-KOH mixture is heated.

SUMMARY AND CONCLUSIONS

The Voges-Proskauer reaction may be recorded, as a presumptive test, 5 hours after the addition of KOH to the test culture.

The Voges-Proskauer and methyl-red reactions seem to be well correlated with the fuchsin-aldehyd test from glucose. On addition of 2 or 3 drops of basic fuchsin, decolorized with sodium sulphite, to

2 c.c. of a 72-hour culture, the Voges-Proskauer negative and methyl-red positive strains gave a very distinct red or violet coloration, while the methyl-red negative and Voges-Proskauer positive group were pink or colorless.

The test for acetyl-methyl-carbinol from sucrose, raffinose, mannitol, and salicin may be accelerated by suitable oxidizing agents, among which the most desirable are hydrogen peroxid, barium peroxid, and potassium dichromate, but such oxidation does not give as good results with galactose or glucose.

If hydrogen peroxid is used, the mixture of the culture and potassium hydroxid should be brought to a boil over the flame or heated in a boiling-water bath for 2 minutes before addition of the oxidizing agent. The addition of an excess of the peroxid produces a transitory color due to its strong bleaching action. About 3 or 4 drops of hydrogen peroxid to 6 c.c. of the KOH-culture mixture gives good results. The pink or eosin-like coloration may be observed in 1 or 2 minutes.